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Published in:
Biochimica et Biophysica Acta %28BBA%29 - Nucleic Acids and Protein Synthesis

DOI:
10.1016/0005-2787(77)90177-0

Publication date:
1977

Biochimica et Biophysica Acta, 478 (1977) 128—145

BBA 99000

THE RESTRICTION FRAGMENT MAP OF RAT-LIVER MITOCHONDRIAL DNA: A RECONSIDERATION

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(Received March 9th, 1977)

Summary

1. Rat-liver mitochondrial DNA (mtDNA) contains at least 8 cleavage sites for the restriction endonuclease Eco RI, 6 for the restriction endonuclease Hind III, 2 for the restriction endonuclease Bam HI and 11 for the restriction endonuclease Hap II.

2. The physical map of the restriction fragments of Eco RI, Hind III, Bam HI and Hap II is constructed on the basis of: (a) the analysis of partially restricted fragments; (b) analysis of the double digests of total mtDNA; (c) the digestion of isolated restriction fragments with other restriction endonucleases; (d) the identification of fragments of complete single and double digestions and of partially digested fragments containing the base sequences complementary to the 12-S and 16-S RNAs of rat-liver mitochondrial ribosomes.

3. The genes for the ribosomal RNAs are shown to be closely linked. This result differs from data previously reported (Saccone, C., Pepe, G., Cantatore, P., Terpstra, P. and Kroon, A.M. (1976) in The Genetic Function of Mitochondrial DNA, pp. 27—36, Elsevier/North-Holland Biomedical Press, Amsterdam).

4. The origin of replication (D-loop) is localized in the vicinity of the small ribosomal RNA gene and the direction of replication is distant from this gene.

5. The mitochondrial tRNA genes are scattered over the genome as in other animal mtDNAs. The approximate minimal number of tRNA genes is 16—20.

6. We concluded previously that the Eco RI restriction fragments A and D are not adjacent and failed to show the overlap of the 16 S rRNA gene for the Eco RI fragment D and Hind III fragment A. This misinterpretation was due to the fact that the two smallest Eco RI fragments could not be detected with the

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Abbreviations: mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; SDS, sodium dodecylsulphate; SSC, 0.15 M NaCl/0.015 M trisodium citrate, pH 7.0.

methods applied and to the lower specific radioactivity of the ribosomal RNAs used in the first series of hybridization experiments.

Introduction

Recently we presented a restriction endonuclease cleavage map of mitochondrial DNA (mtDNA) from rat liver [2]. This map was based on the calculation of the molecular weights of the fragments in complete and partial digests of mtDNA with the restriction enzymes Eco RI and Hind III. The Eco RI fragments of DNA from Φ29 and *Neurospora crassa* mitochondria were used as standards. It was further based on the analysis of double digests of total mtDNA and on the results of digestion of isolated Eco RI fragments with Hind III. There appeared to be a high degree of consistency between the various results. In a subsequent series of experiments we hybridized the mitochondrial ribosomal RNAs to the isolated fragments and observed that the DNA fragments carrying the ribosomal RNA genes were not adjacent but about 4000 basepairs apart [1]. This result appeared exceptional in the sense that other animal mtDNAs tested bear their ribosomal RNA genes on closely linked parts of the genome [3,4]. Inclusion of Bam HI digestion confirmed part of the order of fragments of the map. However, definitive proof of that part of the map containing the ribosomal RNA genes was not obtained in this way, because the two ribosomal RNAs mapped on the same Bam HI fragment. Also the enzymes Hae III, Hin f and Hap II did not settle the question easily, because the number of cleavage sites for these enzymes was quite high. One of the main arguments remained, therefore, the typical pattern of partial digestion products with the enzyme Eco RI. The composition of these partials was inferred from their molecular weights and not analyzed directly. Since the presence of one or more other small fragments could undermine our conclusion, we decided to reinvestigate the matter using the ribosomal RNAs as indicators for the presence of the various fragments in the partial restriction products. The aim of this paper is to present the results of this reinvestigation and to extend the previous observations with data regarding the localization of the origin of mtDNA replication and of mitochondrial tRNAs.

Methods and Materials

Preparation of mitochondrial DNA from rat liver

mtDNA was prepared from the livers of male albino rats (Wistar strain) exactly as described previously [2]. The method is essentially based on the isolation of the closed circular mtDNA by CsCl-ethidium bromide density gradient centrifugation of the lysate of mitochondria prepared by differential centrifugation.

Preparation of mitochondrial RNA from rat liver

55-S mitochondrial ribosomes were isolated as described elsewhere [5], the rRNAs were extracted from the ribosomes with a SDS/phenol procedure. Poly(A)-containing mtRNA was obtained by oligo(dT) cellulose chromatography, as presented in detail recently [6]. Mitochondrial tRNA was prepared as

the 4 S RNA fraction from the total RNA extracted from lysed mitochondria. The method used has been described for the isolation of tRNA from yeast mitochondria [7,8] and has proved useful as well for the isolation of mitochondrial tRNAs from *N. crassa* [9].

Enzymatic fragmentation and gel electrophoresis of mitochondrial DNA from rat liver

The mtDNA was digested at 37° C for 3 h or longer with appropriate amounts of the restriction endonucleases Eco RI, Hind III, Bam HI or Hap II. The latter enzyme was a gift of Dr. J.G.G. Schoenmakers (University of Nijmegen), the former 3 were obtained from New England Biolabs, Beverly, Ma., U.S.A. The media for digestion contained: 100 mM Tris · HCl, 50 mM NaCl, 5 mM $MgCl_2$ and 0.1 mg/ml gelatine for Eco RI; 7 mM Tris · HCl, 60 mM NaCl, 7 mM $MgCl_2$ for Hind III; 6 mM Tris · HCl, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM mercaptoethanol and 0.1 mg/ml gelatine for Bam HI and 6 mM Tris · HCl, 6 mM NaCl, 7 mM $MgCl_2$ and 6 mM mercaptoethanol for Hap II. The pH of these media was 7.6. The final volume of about 50 μl contained 3—5 μg mtDNA. Electrophoresis was performed using composite slabgels of 20 × 30 cm. In most cases the gels consisted of a small sealing layer of 10% polyacrylamide, a layer of about 8—10 cm of 3% polyacrylamide and a 20 cm layer of 0.7% agarose. In some experiments we used longer 10% and 3% polyacrylamide layers and only a small agarose layer to improve the detectability of very short restriction fragments. All further details were the same as described in our previous paper [2].

Iodination of the RNAs

Iodination of the various RNAs was performed according to Getz et al. [10] in a final volume of 50 μl containing 0.1 M sodium acetate (pH 5.0), 62.5 μM KI, 2.3 mM $TlCl_3$, 5—20 μg RNA and 0.5—1.0 mCi of ^{125}I (carrier free 033 L, New England Nuclear). Incubation was for 20 min at 60° C. After chilling to 0° C 1 μl of 0.1 M Na_2SO_3 and 50 μl ammonium acetate (pH 9.3) were added and the incubation was continued for 20 min at 70° C. The ^{125}I-labelled RNA was purified by the procedure of Prensky et al. [11], slightly modified as described elsewhere [9,12].

Hybridization procedure

The restriction fragments were denatured and transferred onto nitrocellulose filterstrips (Sartorius, 0.45 μm pore size) exactly as described by Southern [13]. The filters were baked at 80° C for 4 h. Hybridization was performed either in a vessel similar to that described by Southern [13] or in a glasstube of 200 mm length and a diameter of 6 mm. In the latter case the filter was inserted gently, one end was closed with a plastic stopper, the hybridization mixture (2.5 ml 3 × SSC, 0.2% SDS) was introduced, the wet filter was pressed down against the wall, the other end of the tube closed and the whole tube clamped tightly in a glass tray in a horizontal position. The whole assembly was submerged in a waterbath at 65° C with the axis in the shaking direction. The hybridization time was 19 h; the input DNA per gel slot was approx. 3 μg. After the hybridization the filters were washed three times with 2 × SSC at 25° C, once with 2 × SSC at 60° C and then treated with a mixture of 10 μg/ml

DNAase-free RNAase A and 25 units/ml RNAase T1 for 1 h at 20°C. After the RNAase-treatment the filters were washed again three times with 2 × SSC at 25°C, dried and finally subjected to autoradiography for various times. The autoradiograms were aligned with a true size photograph of the gel. In some experiments the radioactivity associated with the various fragments was quantified by liquid scintillation counting after cutting the stripfilters into pieces.

Fragment nomenclature

The terminal fragments are designated by capital letters in order of increasing electrophoretic mobility. In combination with the fragment designation letter, the following abbreviations have been used for the restriction enzymes: E for Eco RI, H for Hind III, B for Bam HI and Hap for Hap II. The new fragments in double digests are designated by arabic numerals in order of increasing electrophoretic mobility preceded by the combined abbreviations of the endonucleases used. Partial digestion products are designated by the abbreviation of the enzyme used, followed by the letter p and an arabic numeral in order of increasing electrophoretic mobility. This nomenclature was also used in our previous papers on rat-liver mtDNA [1,2].

Electron microscopy

(A) Restriction endonuclease fragments were made after glyoxal fixation of the D-loop as described by Brown and Vinograd [14]. These fragments were prepared for electron microscopy by a formamide modification of the basic protein film technique [15]. The spreading solution contained 2 μg/ml of DNA, 60% formamide, 0.4 M ammonium acetate, 8 mM EDTA (pH 8.3) and 0.01% cytochrome *c*. For the spreading distilled water, redistilled twice over quartz, was used as hypophase.

The protein nucleic acid film was picked up on 400 mesh copper grids covered with parlodion. Grids were dehydrated and stained by dipping into $5 \cdot 10^{-2}$ mM uranyl acetate, $5 \cdot 10^{-2}$ mM hydrochloric acid in 90% ethyl alcohol for 30 s, followed by a brief rinse in isopentane. Finally they were rotary shadowed with platinum at an angle of 5°.

(B) The DNA-fragments cleaved by the restriction endonuclease Hap II (Fig. 7) were spread by absorption to charged carbon films and rotary shadowed with Pt at an angle of 5° as decribed by Brack et al. [16].

The specimens were examined in a Philips EM200. Pictures were taken at magnifications 5000—17000 on 35 mm Kodak FRP film. Calibration of magnification was done with a grating replica (Ladd. 2160 lines/mm). Length measurements were made with a Hewlett-Packard 9864A Digitizer connected to a 9820A calculator system. Full-length mtDNA molecules were added after enzymatic restriction as internal size standards.

Results and Discussion

Stripfilter hybridization of mtDNA fragments with 16 S RNA of mitochondrial ribosomes

The sedimentation values of the two large ribosomal RNAs of the ribosomes from animal mitochondria are 16 and 12 S [5]. Stripfilters were prepared con-

taining the fragments of complete and partial digests of mtDNA treated with the various restriction endonucleases. It was hoped that the method of strip-filter hybridization [13] should be useful and sensitive enough to indicate also the partials containing the genes of either of the two ribosomal RNAs. The results of a number of these experiments are shown in Fig. 1. In this and in some of the further figures the ultraviolet photographs of the original slabgels are combined with the autoradiograms of the stripfilters after hybridization with the ^{125}I-labelled RNAs. Fig. 1A shows that the Hind III fragments A and B contain sequences complementary to the 16 S rRNA. The relative amount of radioactivity bound to the fragment HB was 3 times higher than that bound to the fragment HA. Of the Hap II fragments (Fig. 1B) radioactivity was bound at the position of HapB, HapE and HapH. It should be noted that the HapH band contains 2 fragments of the same electrophoretic mobility. The only Eco RI fragment, binding the 16 S rRNA, was ED (Fig. 1C). The conclusion of these

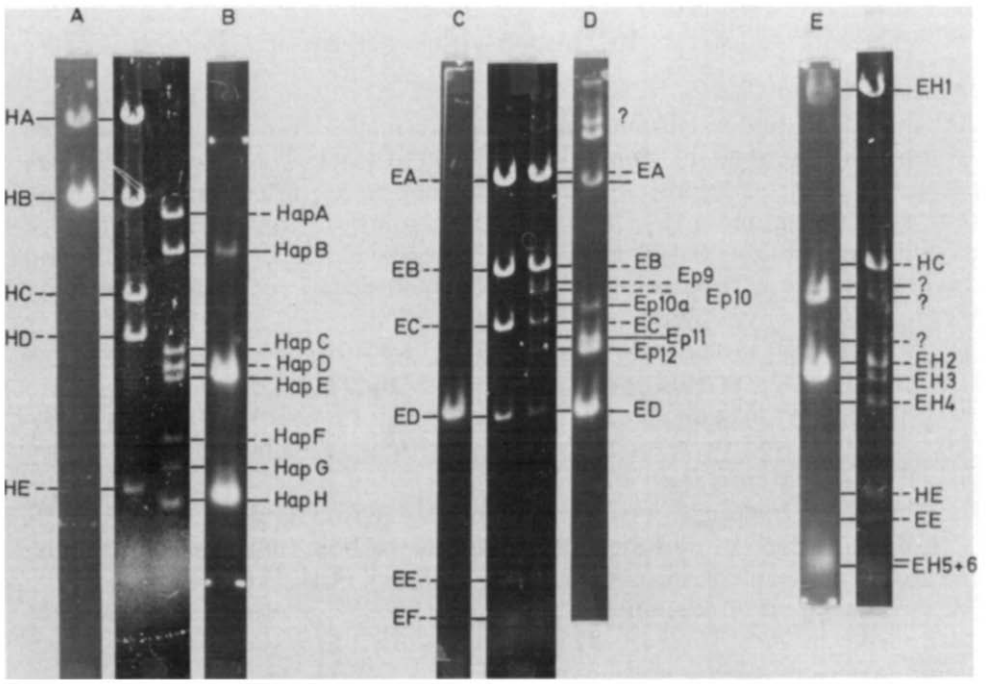

Fig. 1. Hybridization of mitochondrial 16 S rRNA with restriction fragments of mtDNA. Stripfilters containing the denatured restriction fragments of rat-liver mtDNA were incubated with ^{125}I-iodinated 16 S rRNA from rat-liver mitochondrial ribosomes. For details, see the Methods section. The specific activity of 16 S rRNA was $1.4 \cdot 10^6$ cpm/μg. The input was $0.56 \cdot 10^6$ cpm per filter. Divided over the various fragments, between 1.5 and 2.5% of the input counts were bound to the filters. Photographs of the autoradiograms aligned with true size photographs of the original gels are given. The autoradiograms are indicated with the capital letters. The positions of the various fragments visible on the original gels and autoradiograms are indicated by the small black lines. Solid lines: bands present on both gel and autoradiogram; broken lines: bands present only in gel. The length of the various fragments is given in the Tables I and II. Questionmarks indicate the positions of bands of partial digestion products, which are not further characterized. A, hybridization with a Hind III digest; B, hybridization with a Hap II digest; C, hybridization with an Eco RI digest; D, hybridization with an incomplete Eco RI digest; E, hybridization with a double digest of Eco RI and Hind III.

experiments is that the Hind fragments A and B are adjacent and that the cleavage site between these 2 fragments is located on Eco RI fragment D. In the same area the Hap II fragments B, E and H (one or both) are situated. In Fig. 1D the hybridization pattern of 16 S rRNA and the fragments of a partial digest with Eco RI are shown. The partials previously indicated as Ep11 and Ep12 (2) bind 16 S rRNA and do, therefore, contain ED. Ep10 is not labelled, but radioactivity is found at a position between EC and Ep10. This partial digestion product was not previously detected and interpreted as such. Another interesting feature is the presence of an ED containing partial at the position of EA: it is clearly not EA itself that is labelled as can be concluded from Fig. 1C.

To substantiate the conclusion that Eco RI fragment D contains a Hind III cleavage site hybridization was performed using a stripfilter containing the fragments of mtDNA digested with both Eco RI and Hind III (Fig. 1E). The fragments EH3 and EH6 (not previously detected) are labelled. These fragments derive from HB and ED and from HA and ED respectively. This was shown in separate experiments (cf. Table II). The bands just below HC are considered to be the partial digestion products comparable to Ep11 and Ep12. The data obtained with double digests of Eco RI or Hind III with Bam H1 are not shown. The larger part of the HA fragment (HB1, cf. Table II) and the smaller fragment of EA (EB2, cf. Table II) contained the sequence complementary to the 16 S rRNA.

The findings presented above are different from those presented earlier [1,2]. In the previously published [1] map the Hind III fragments A and B were separated by the fragment HD. If we reconsider our earlier hybridization data, it can be seen that the fragment HA has indeed bound some RNA but in the system used at that time the binding was not significantly different from the background. The procedure of iodination used in the present study, however, led to much higher specific radioactivity of the RNAs. A strong argument for the previous ordering of fragments was the pattern of partial digestion products with Eco RI. We have now detected still another partial digestion product with a lower molecular weight than EB (designated Ep10a in Fig. 1D). The only reasonable manner to explain the high number of partials smaller than EB is to assume that more than two small Eco RI fragments have to be present. Once one accepts this assumption it is clear that the reasoning for the previous ordering of fragments becomes invalid.

The presence of more than two small Eco RI fragments

As outlined in the preceeding paragraph, the only possible explanation for the results of the stripfilter hybridization experiments with 16 S rRNA implicates the presence of more than 2 small Eco RI fragments. By overloading the gelslots and by using a different type of composite slabgel we were, indeed, able to detect two smaller fragments with a length of less than 150 basepairs each. With the same technique the fragment HF and the fragment HapI and HapJ could be visualized. The existence of HF was previously deduced from the presence of a partial digestion product consisting of HE plus HF [2]. Photographs of the gels containing these smaller fragments are shown in Fig. 2. When the restriction endonucleolytic digestion was incomplete, we were able to detect faint bands of partial digestion products as well. These are shown in Fig.

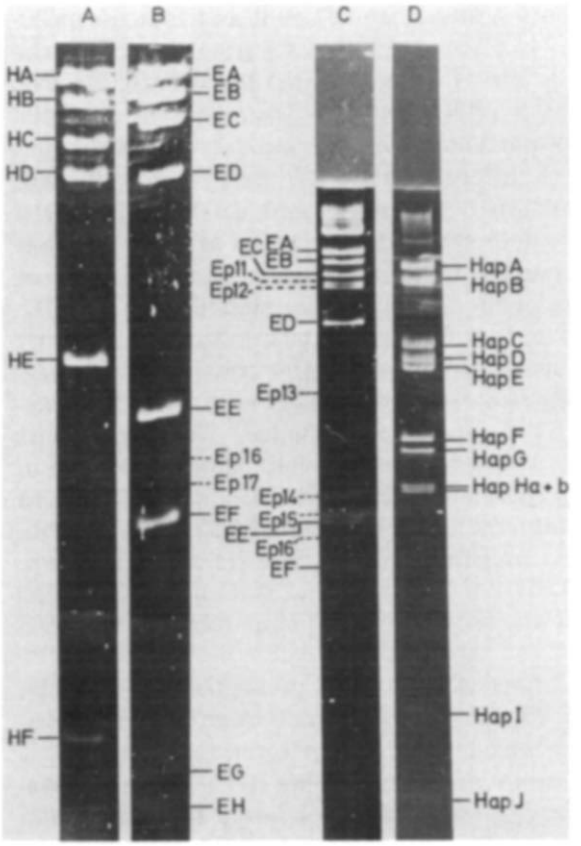

Fig. 2. Slabgel electrophoreses of restriction fragments of mtDNA. The composition of the gels was as follows: a layer of 10—15 cm of 10% polyacrylamide, a layer of 15—10 cm of 3% polyacrylamide and a layer of about 5 cm of 0.7% agarose. To detect small fragments the gels were loaded with 20—25 μg mtDNA per slot. A and B are parts from the same gel, C and D are taken from two different gels. The position of the various fragments is indicated with black lines; the interrupted lines indicate the position of partial digestion products. For further details see the Methods section and ref. 2. A, a Hind III digest; B and C, Eco RI digests; D, a Hap II digest.

2B and C. The fragment lengths of the smaller fragments were roughly estimated by extrapolation, using the fragments EE, EF, HE and HF as the markers. In this way we arrived at lengths of 120 and 100 basepairs for the fragments EG and EH, respectively. The pattern of partials was consistent with these molecular weights and with a clustering of these fragments in one area of the genome, in between the Eco RI fragments D and C. The most likely order in this cluster is EHFG. The inferred composition of the partials indicated (Fig. 2B and C) is as follows: Ep13: EE + EF + EG + EH; Ep14: EE + EH; Ep15: EF + EG + EH; Ep16: EF + EG; Ep17: EF + EH. The order of all Eco RI fragments becomes, than: ADEHFGCBA.

The lengths of all fragments obtained by single and double digestion of rat-liver mtDNA with the four restriction enzymes are summarized in Tables I and II. The data of double digestion are obtained in two ways. In the first place total mtDNA was incubated with two different enzymes in two steps of incuba-

TABLE I

LENGTH OF THE RESTRICTION ENZYME FRAGMENTS OF RAT-LIVER MITOCHONDRIAL DNA

For experimental details and nomenclature see the Methods section.

Bam HI fragments			Eco RI fragments		
	M X 10^{-6}	Basepairs		M X 10^{-6}	Basepairs
BA	6.65	10 000	EA *	3.64	5 500
BB	2.96	4 450	EB *	2.36	3 550
	9.61	14 450	EC *	1.76	2 650
			ED *	1.20	1 800
Hap II fragments			EE	0.43	650
			EF	0.27	400
	M X 10^{-6}	Basepairs	EG	0.08	125
HapA	2.32	3 500	EH	0.07	100
HapB	1.89	2 850		9.81	14 775
HapC	1.16	1 750			
HapD	1.08	1 630	Hind III fragments		
HapE	1.03	1 550		M X 10^{-6}	Basepairs
HapF	0.74	1 120			
HapG	0.64	970	HA *	3.96	5 950
HapHa	0.45	680	HB *	2.50	3 750
HapHb	0.45	680	HC *	1.53	2 300
HapI	0.12	180	HD *	1.26	1 900
HapJ	0.07	110	HE	0.53	800
	9.95	15 020	HF	0.10	150
				9.88	14 850

* Fragments containing tRNA genes (see text).

tion without isolation of the various fragments. In the second place the fragments of an incubation with one enzyme were separated on gels and reisolated for digestion with the second enzyme. For this purpose we used slabgels with large 3% polyacrylamide layers, because in our hands the method of fragment extraction from polyacrylamide with buffer as used by Van den Hondel and Schoenmakers [17] gave a better yield of fragments than the other methods tried [18,19].

The localization of the 12 S ribosomal RNA gene

We have shown previously [1], that the gene for the 12 S rRNA of mitochondrial ribosomes is localized on a part of the genome that is shared by the fragments A of Eco RI, Hind III and Bam HI. This observation could be confirmed with the stripfilter hybridization approach. With the 12 S rRNA we met the problem of contamination of the RNA preparation with fragments of 16 S rRNA. In most of the experiments 16 S rRNA was added as competitor RNA. Fig. 3 shows the gels and autoradiograms of 12 S rRNA hybridized to filters containing the mtDNA fragments after digestion with Eco RI, Hind III and Hap II. In the experiments with Hind III and Hap II no competitor 16 S rRNA was present in these cases. The main hybridization is with the fragments HA (Fig. 3B) and HapE (Fig. 3C). In separate experiments (not shown) it was observed that the 12 S rRNA hybridized with fragment Bam A. From experiments

TABLE II

LENGTH OF THE NEW RESTRICTION FRAGMENTS OF RAT-LIVER MITOCHONDRIAL DNA OBTAINED IN DOUBLE DIGESTS WITH DIFFERENT ENDONUCLEASES

Rat-liver mtDNA was digested in two successive incubations with a combination of two of the restriction enzymes Eco RI, Hind III, Bam HI and Hap II. The restriction fragments of either enzyme, not cleaved by the other are not given in this table. In a separate series of experiments the restriction fragments were isolated after digestion with the first enzyme and subsequently further digested with a second enzyme. For further details and nomenclature see the Methods section.

Eco RI + Bam HI fragment	basepairs	Hind III + Bam HI fragment	basepairs	Bam HI + Hap II fragment	basepairs
EB1 *	1950	HB1 *	3050	BHap1	3100
EB2 *	2600	HB2 *	2900	BHap2	1220
EB3 *	1950	HB3	1550	BHap3	540
EB4	1650	HB4 *	920	BHap4	430
EB1 + EB2 = EA^+		HB1 + HB2 = HA^+		BHap1 + BHap4 = HapA	
EB3 + EB4 = EB^+		HB3 + HB4 = HC^+		BHap2 + BHap3 = HapC	
EB1 + EB4 = BB^+					

Eco RI + Hind III fragment	basepairs	Eco RI + Hap II fragment	basepairs	Hind III + Hap II fragment	basepairs
EH1 *	5450	EHap1	2050	HHap1	1360
EH2 *	1450	EHap2	1500	HHap2	1320
EH3 *	1400	EHap3	1360	HHap3	1270
EH4 *	1250	EHap4	1360	HHap4	950
EH5	370	EHap5	900	HHap5	275
EH6	350	EHap6	410	HHap6	160
		EHap7	260		
EH1 + EH6 = HA^+				HHap3 + HHap5 = $HapE^+$	
EH2 + EH5 = HD^+		EHap1 + EHap2 = $HapA^+$		HHap4 + HHap6 = $HapF^+$	
EH2 + EH4 = EC^+		EHap4 + EHap7 = $HapD^+$		HapA contains HHap1, HHap2 and HE^+	
EH3 + EH6 = ED^+		HapB contains EE, EF, EHap3 and $EHap6^+$			
		HapE contains EHap5 and a fragment of 680 bp^+			

+ Independently confirmed by digestion of isolated terminal restriction fragments.
* Fragments containing mtRNA genes.

with double digests of Bam H1 with either Hind III or Eco RI in the presence of competitor, it could be concluded that the 12 S rRNA hybridized exclusively with the larger part of HA (HB1, cf. Table II) and the smaller part of EA (EB2, cf. Table II). This observation is compatible with the conclusion reached above, that the cleavage site between EA and ED lies on HA. In the cases of Hind III and Hap II (Fig. 3B and C) we need some correction for the contributions of (fragments of) contaminating 16 S rRNA. Comparison with Fig. 1A and B shows that the fragments labelled are the same (HA and HB; HapB, HapE and HapH). The amount of radioactivity bound to the various fragments was quantitated. The radioactivity found on 12 S rRNA-stripfilters was corrected for 16 S rRNA contamination assuming that all counts bound to HB and HapB were to be ascribed to the 16 S rRNA contaminants and further assuming the same relative contribution of the contaminants to all labelled fragments. In both cases this led to the conclusion that about 50% of the counts were due to 16 S tRNA contamination. It further revealed that the fragments HapE and HapH contain sequences complementary to the 12 S rRNA, about 5/6 of the gene lying on

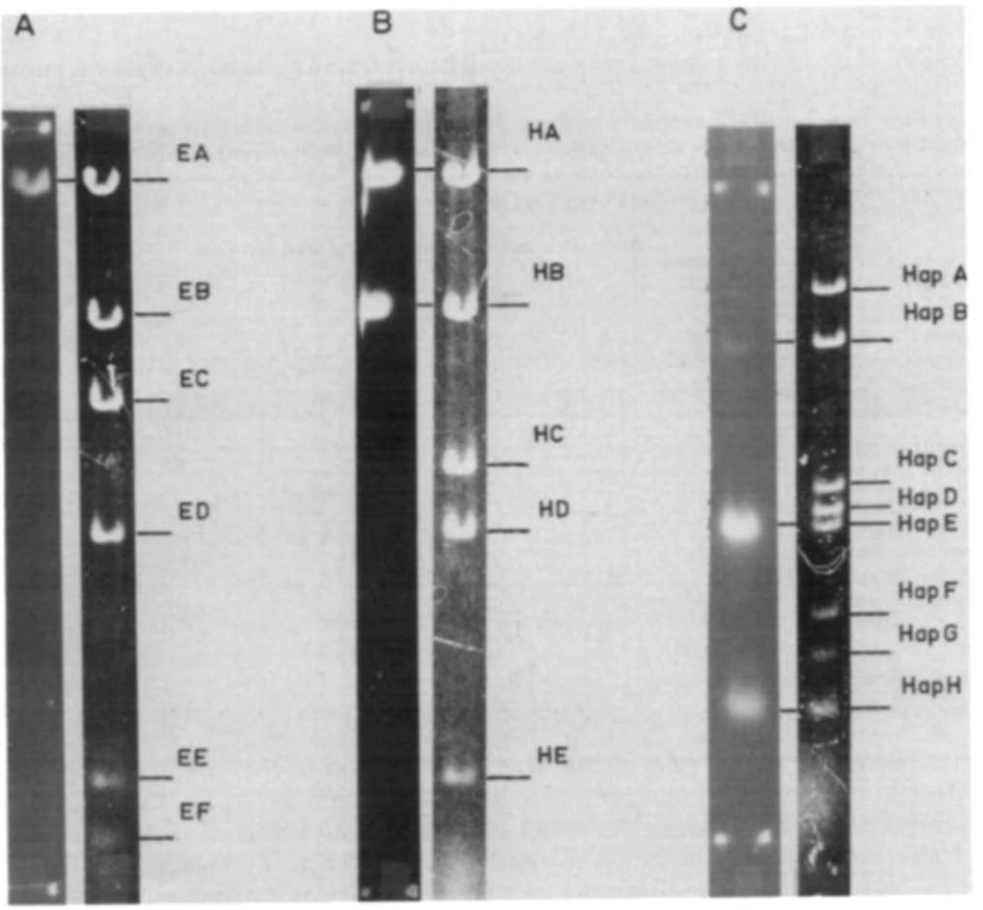

Fig. 3. Hybridization of mitochondrial 12 S rRNA with restriction fragments of mtDNA. For details see the Methods section and the legend to Fig. 1. The specific activity of the 12 S rRNA was 128 500 cpm/μg (a) and 156 000 cpm/μg (B and C). The input was 44 000 cpm (A) and 140 000 cpm (B and C) per filter. 1.1, 11 and 2.5% of the input counts were bound to the filters in A, B and C, respectively. 10-fold excess competitor 16 S rRNA was present in the experiment illustrated in A, but not in those of B and C. Photographs of the autoradiograms (indicated by the capital letters) aligned with true size photographs of the original gels are given. A, hybridization with an Eco RI digest; B, hybridization with a Hind III digest; C, hybridization with a Hap II digest.

HapE. Since HapE also contains about half of the 16 S rRNA gene it follows that the two ribosomal RNA genes are very closely linked on the mtDNA, leaving a gap in between the two rRNA cistrons of only about 200 basepairs. The data for this calculation are given in Table III. Taking the number of basepairs of HapH as 44% of the length of the 16 S rRNA we arrive at a molecular weight equivalent to 1500 ribonucleotides which is close to the value of $0.52 \cdot 10^6$ obtained by Kleinow [20] for Locusta mt-ribosomes and of $0.54 \cdot 10^6$ by Hamilton and O'Brien [21] for bovine mt-ribosomes, but lower than the value of $0.65 \cdot 10^6$ reported by Groot et al. [22]. The calculated space occupied by the 16 S rRNA on the mtDNA by this method is in excellent agreement with electron microscopical observations on mtDNA · mtRNA hybrids [3,4].

TABLE III

QUANTITATION OF THE STRIPFILTERHYBRIDIZATION WITH THE 16-S AND 12-S RNAs FROM MITOCHONDRIAL RIBOSOMES

Some of the stripfilters shown in Figs. 1 and 3 were cut in pieces and assayed in toluene containing 0.4% PPO and 0.01% POPOP in a liquid scintillation counter. Regions of the filters without DNA were used to obtain background values. The radioactivity in areas with labelled bands was corrected for this background. For further details, see the legend to Figs. 1 and 3, the Methods section and the text.

	Restriction fragments hybridizing with mtrRNA					
	HA	HB	HapB	HapE	HapHa	HapHb
Fragment length [1] in basepairs	5950	3750	2850	1550	680	680
16 S rRNA (Fig. 1A and 1B)						
% hybridization (p)	24	76	5	51	44	0
$p/l \times 10^3$	n.r.	n.r.	2	33	65	n.r.
12 S rRNA (Fig. 3B and C)						
% hybridization						
before correction for contaminating 16 S rRNA	61	39	2.5	68	←29.5→	
after correction for contaminating 16 S rRNA	49(100)	0	0	42(84)	0	8(16)

n.r. = not relevant.

The origin and direction of replication: a confirmation of the fragment map

After having localized the genes for the 2 risobomal RNAs on the mtDNA, we were interested to search for other markers. Using standard techniques we were able to show that the origin of replication, visible as the D-loop, was localized on the fragment EA (Fig. 4A) and HA (Fig. 4B). It further appeared that the D-loop overbridged a region of the mtDNA containing a cleavage site for Hap II. Typical H-form molecules as described by Brown and Vinograd [14] were found in the Hap II digest (Fig. 4D). The D-loop was also found in partial digestion products of Hind III (Fig. 4C), Eco RI (not shown) and Hap II (Fig. 4E and 4F). From length measurements of a large number of different molecules it was concluded that the two Hap II fragments present in H-form molecules are HapC and one of the HapH fragments. By digestion of isolated Bam-, Eco- and Hind-fragments with Hap II we knew already that one of the Hap H fragments was localized on BA/EA/HA and the other on BA/ED/HB. Since the fragments HapC and HapA are cleaved by Bam H1 we coulc decide that the D-loop is localized quite close to the rRNA genes. In fact either the origin or the growing replication fork reside on the same fragment (HapHb) as part of the 12 S rRNA gene. The left hand parts of the EA and HA fragments illustrated in Fig. 4A and 4B represent the parts of the mtDNA that contain the sequence compelentary to the 12 S rRNA. We further looked for molecules containing expanding D-loops. In Fig. 5 two HA fragments containing D-loops of different length are shown. It can be seen that the left hand parts are constant and the right hand parts are variable in length. The direction of replication is, therefore, from left to right, off from the region containing the ribosomal RNA genes. The fragment HapHb, therefore, contains the origin of replication,

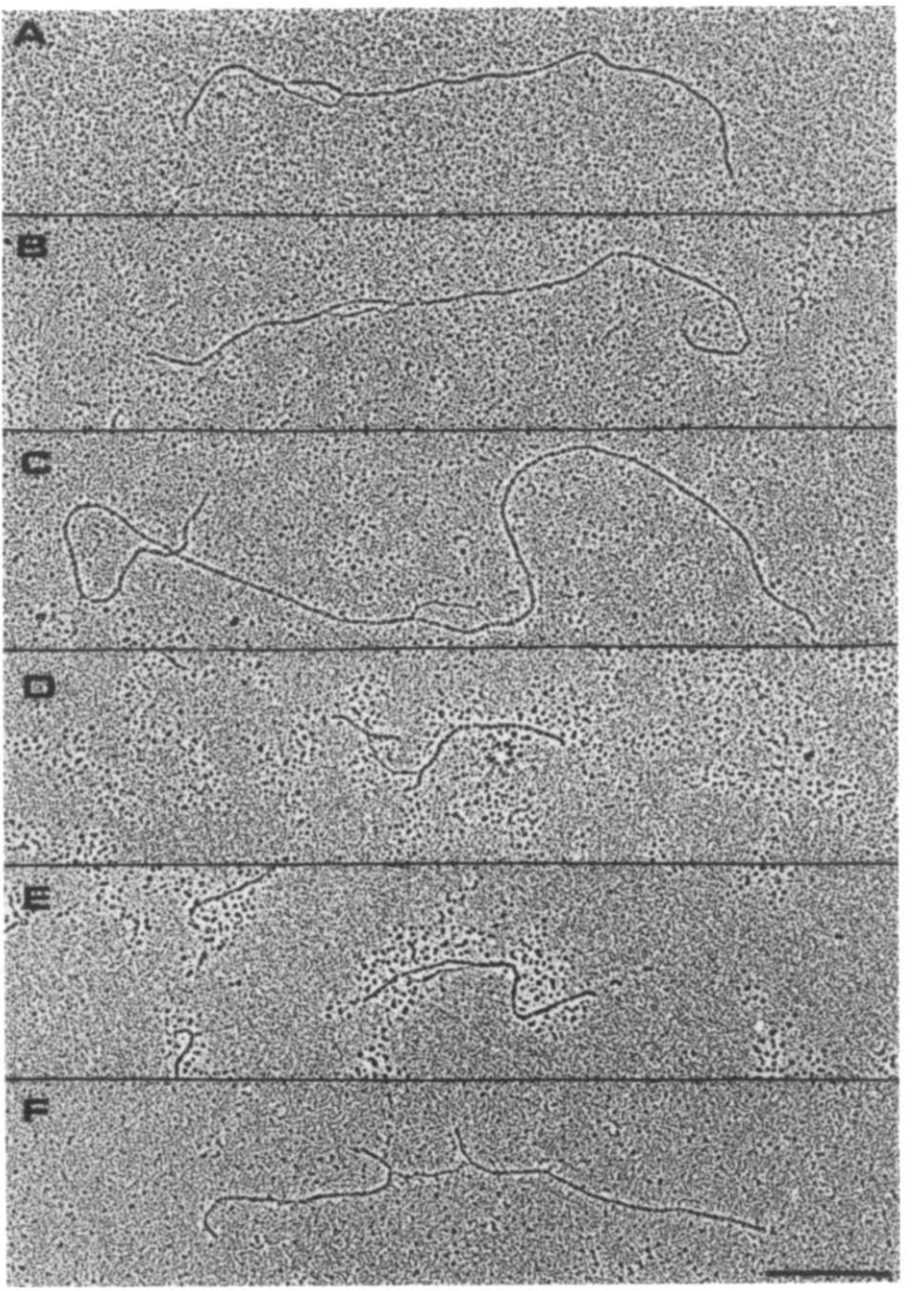

Fig. 4. Electron micrographs of mtDNA fragments bearing a D-loop. Details of the treatment of the mtDNA for electron microscopy are given in the Methods section. The magnification is the same for all molecules depicted. The bar is 0.5 μm. A, fragment EA; B, fragment HA; C, a partial digestion product of Hind III; D, the fragments HapC and HapH, interconnected by the single DNA strand of the D-loop (cf. ref. 14); E, a partial digestion product of Hap II with the length of HapC plus HapH; F, 2 partial digestion products of Hap II, interconnected by the single DNA-strand of the D-loop.

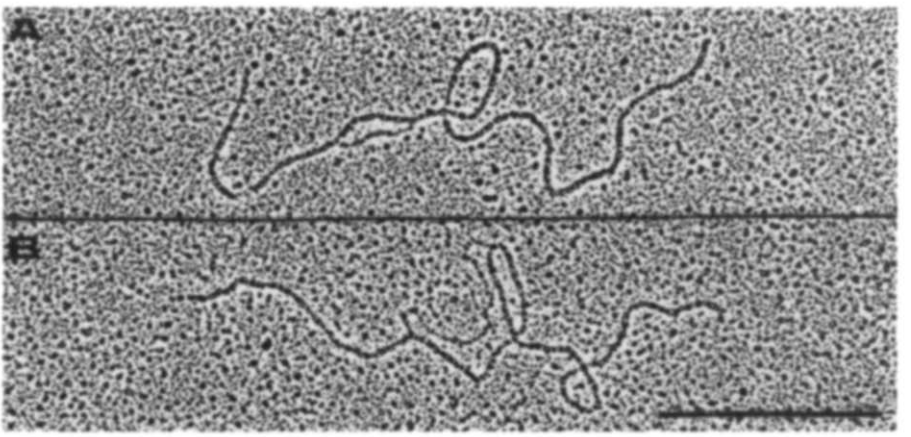

Fig. 5. The expanding D-loop of rat-liver mtDNA. Two HA fragments are shown bearing D-loops of different length. The magnification is the same for A and B. The bar is 0.5 μm.

the fragment HapC the part of the D-loop that will act as the growing replication fork, if replication proceeds.

Using the D-loop as a marker we have constructed the physical map of Hind III fragments by measuring the length of a large number a complete and partial digestion products, all containing the D-loop. The results are shown in Fig. 6. The only order of Hind III fragments compatible with the results of these measurements is ABDC(EF)A. This order is the same as the order arrived at by means of the fragment analyses and hybridization experiments reported above.

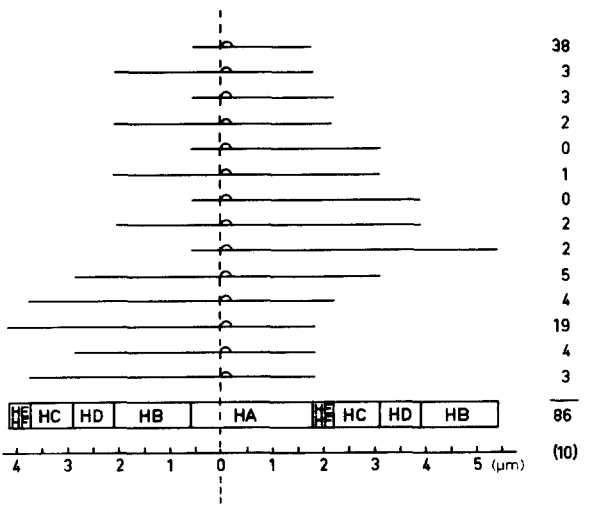

Fig. 6. The order of Hind III fragments of rat-liver mtDNA as based on electron microscopical length measurements. mtDNA was treated with glyoxal and partially digested with Hind III. Molecules containing D-loops were measured. The figure shows the best fitting mutual relation between the molecules of various length. The resulting fragment order is indicated at the bottom part of the figure. The column at the right indicates the number of molecules scored for each length category. Of two theoretically possible length classes, no example was found. The number 10 between brackets indicates the number of molecules not fitting the pictures. Most of these were molecules broken very close to the D-loop.

The method is not sensitive enough to decide on the order of HE and HF relative to HA and HC. This sequence was already known from previous fragment analyses [2]. A similar approach for the construction of the restriction fragment map has been used by Upholt and Dawid for sheep and goat [23]. Recently also Koike et al. [24] have published the results of such an approach for the Eco RI fragments of rat-liver mtDNA. The fragment order is identical to the one presented in this paper. Unfortunately the details of their work are neither described in their paper nor presented at the conference to the proceedings of which the paper belongs. The fragments EG and EH were not detected by these authors.

The construction of the restriction fragment map of Hap II fragments

Complete digestion of rat-liver mtDNA with the endonuclease Hap II gave rise to 8 bands on standard agarose-polyacrylamide gels. In this pattern the fragment HapH was clearly present twice on a molar basis. By using gels with a 10% polyacrylamide layer and by overloading these gels another two bands representing smaller fragments were detected. Furthermore, the two HapH fragments can be separated under these conditions (see Fig. 2D). Also in the preparations used for electron microscopical length measurements, a large number of fragments smaller than HapH could be detected as can be seen in Fig. 7. We estimate the length of these fragments in the order of 100 and 200 basepairs. With the exception of these two smaller fragments we have been able to construct the map of the Hap II fragments as well. The map is based on the hybridization and electron microscopical experiments described above and further

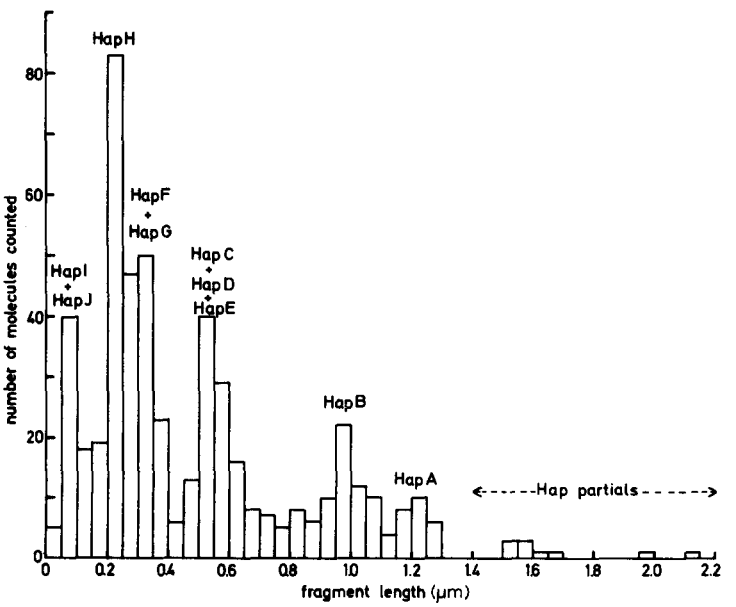

Fig. 7. Length distribution of the fragments of rat-liver mtDNA after endonucleolytic digestion with Hap II. Method B (see Methods section) was used for spreading the fragments. A total of about 400 molecules was scored. The fragment length was calculated relative to the length of open circular molecules. 1 μm was equivalent to 2980 basepairs under these experimental conditions.

on the digestion of isolated Bam H1, Eco RI and Hind III fragments with Hap II. The data are included in Tables I and II. The order of fragments is: ADFBHaEHbCGA.

Stripfilter hybridization of mtDNA fragments with mitochondrial tRNAs and poly(A)-containing RNA

The 4 S RNA fraction from rat-liver mtRNA was isolated and iodinated with ^{125}I. Stripfilter hybridization was carried out in the presence of excess mitochondrial rRNAs as competitor. The results are shown in Fig. 8. On the filters containing the fragments of single digests as well as on those with the fragments of double digests most of the fragments coincide with spots on the autoradiograms. It is clear that fragment ED (Fig. 8A) is very weakly labelled. The smallest amount of radioactivity bound to any fragment of the single and double digests with Eco RI, Bam HI and Hind III corresponded to about 5—6% of the total radioactivity bound. Assuming an even distribution of the iodine-label over the various tRNAs, and the presence of the various tRNAs in about equimolar amounts, the lower estimate for the number of tRNA genes on rat-liver mtDNA is in the order of 16—20. This is in good agreement with the number of tRNA genes so far recognized on the mtDNA from other animal sources [3,4].

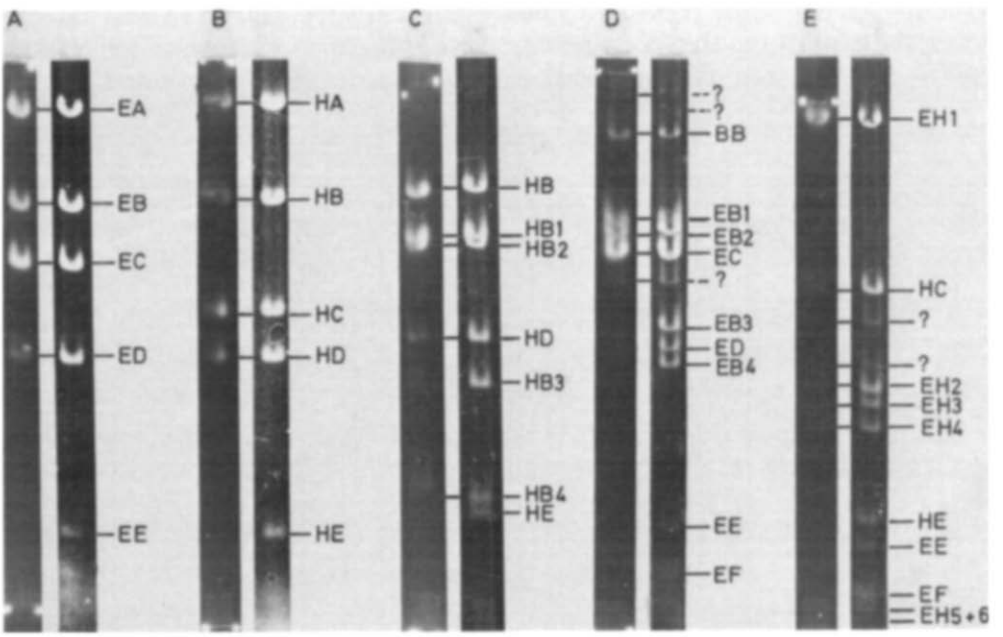

Fig. 8. Hybridization of total mitochondrial tRNA with restriction fragments of mtDNA. For details see the Methods section and the legend to Fig. 1. The specific activity of the tRNA preparation was 275 000 cpm/μg. The input was 180 000 cpm per filter. About 0.4% of the input counts were bound to the filters. Corrections were made for the background of about 10 cpm per mm filterlength. 15-fold excess competitor 16 S + 12 S rRNA was present in the hybridization medium. Photographs of the autoradiograms (indicated by the capital letters) aligned with true size photographs of the original gels are given. A, hybridization with an Eco RI digest; B, hybridization with a Hind III digest; C, hybridization with a double digest of Hind III and Bam H1; D, hybridization with a double digest of Eco RI and Bam HI; E, hybridization with a double digest of Eco RI and Hind III.

The fragments that were found to be able to bind tRNAs in the presence of competitor rRNA are marked with an asterisk in Tables I and II. As a general conclusion it appears that, also on mtDNA of the rat, the tRNA genes are scattered on the mitochondrial genome.

A similar random distribution was found for the poly(A)-containing mitochondrial RNA. In this respect the stripfilter hybridization confirmed our previous data [1]. However, if mitochondrial rRNAs and tRNAs were added as competitors, it was observed that the binding of the poly(A)-containing RNA to the fragments containing the rRNA-complementary sequences was considerably reduced. This was specially clear for fragment ED. Although contamination with these RNAs may offer a simple and seemingly straightforward explanation, it may be recalled that mitochondrial transcription is thought to be a symmetrical process initially giving rise to large transcription products [25,26], which may well contain the ribosomal RNA cistron and, moreover, a stretch of poly(A) at their 3′ end [4,27]. These alternatives as well as the precise localization of specific mt-tRNAs are presently under investigation.

Concluding remarks

The data presented in this paper are summarized in Fig. 9. Our data show that it is very difficult to rely on the inferred compositions of partial digestion products. This is especially true if the conditions of electrophoresis are such

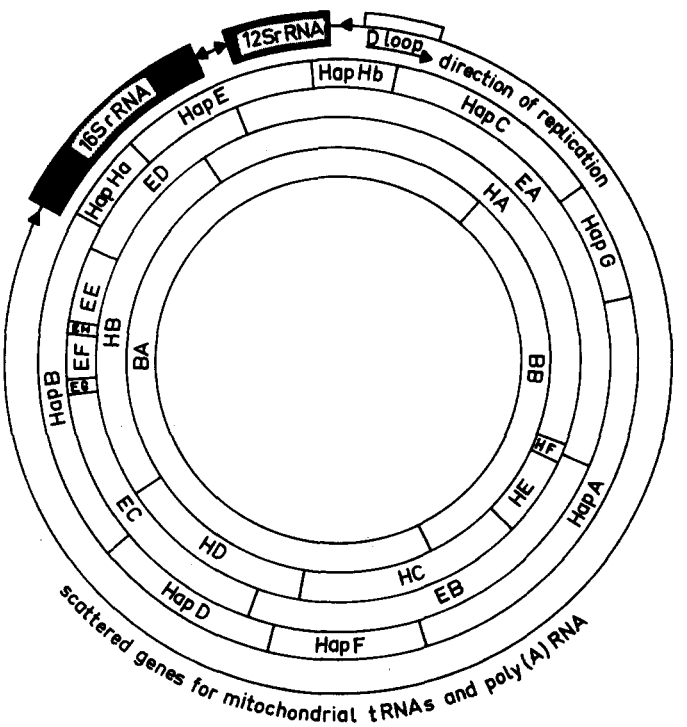

Fig. 9. The restriction fragment map of rat-liver mtDNA and the position of a number of genetic markers on this map.

that small fragments and some partials can be missed or overlooked. This occurred to us in our original study, although at that time we already found 6 fragments with the enzymes Eco RI and Hind III, a number about twice as high as described at that time for mtDNA of other animal species [14,28]. However, Upholt and Dawid have described the presence of 5 cleavage sites for Eco RI in mtDNA of a goat [23]. Also Koike et al. have recently raised their number of Eco RI fragments of rat-liver mtDNA, in their case from 5 to 6 [24]. Unfortunately the gels with their partial digests, on which their experiments are partly based, have not yet been published. Similar movements of fragments in the fragment maps of mtDNA have also been made for yeast by Sanders et al. [29] and *N. crassa* by Küntzel et al. [30,31].

In conclusion, the combined approach of electrophoretic restriction fragment analysis, stripfilter hybridization with marker RNAs, and electron microscopical length measurements has led to a consistent physical characterization. It has further revealed possibilities for the isolation of interesting parts of the mitochondrial genome. It is especially intriguing to further characterize the origin of replication. This is quite well feasible because the Hap Hb fragment can be easily obtained by Hap digestion of Hind III fragment A or by separation from HapHa on 10% polyacrylamide gels. This fragment as it contains a replication point may well be interesting also from the viewpoint of genetic engineering.

Acknowledgements

These studies were supported in part by a grant to AMK from the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO) and by a grant to CS from the Consiglio Nazionale delle Ricerche (CNR), Italy. The authors are indebted to Dr. Annika Arnberg for interest and advice, to Theo Deddens for drawing the figures and to Bert Tebbes and Klaas Gilissen for the photography.

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